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A Low-CO_2-Inducible Gene Encoding an Alanine:α-Ketoglutarate Aminotransferase in *Chlamydomonas reinhardtii*[1]

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At low-CO_2 (air) conditions, the unicellular green alga *Chlamydomonas reinhardtii* acquires the ability to raise its internal inorganic carbon concentration. To study this adaptation to low CO_2, cDNA clones induced under low-CO_2 growth conditions were selected through differential screening. One full-length clone is 2552 bp, with an open reading frame encoding 521 amino acids. The deduced amino acid sequence shows about 50% identity with alanine:α-ketoglutarate aminotransferase (Ala AT, EC 2.6.1.2) from plants and animals, and the mRNA of this clone increased 4- to 5-fold 4 h after cells were switched from high-CO_2 to low-CO_2 growth conditions. The expression of the enzyme and its activity also increased accordingly at low-CO_2 growth conditions. To study the physiological role of Ala AT, a pyridoxal phosphate inhibitor, aminooxyacetic acid, was added at 40 μM to the growth medium when cells were beginning to adapt to low CO_2. This caused a 30% decrease in the maximum photosynthetic rate in air-adapting cells 8 h later. The addition of the inhibitor also caused the cells to excrete glycolate, a photorespiratory intermediate, but did not change the apparent affinity of the cell for external CO_2. These physiological studies are consistent with the assumption that Ala AT is involved in the adaptation to low-CO_2 conditions.

Many algae have evolved a mechanism to raise their internal CO_2 concentration to adapt to low-CO_2-containing aquatic environments. Cells with this CCM acquire an ability to accumulate inorganic carbon (CO_2 and HCO_3^-) to levels higher than what can be obtained by simple diffusion. The CCM results in increased internal CO_2, which favors the carboxylation reaction of Rubisco over the competitive oxygenation reaction (Badger et al., 1980; Aizawa and Miyachi, 1986). As a result, algae with the CCM can grow on very low-CO_2 concentrations, lower than what can be tolerated by plants with C3-type photosynthesis. However, even though the CCM is present in algae, there is still a significant metabolic flux through the C2 cycle when cells are grown at atmospheric levels of CO_2 and O_2 (Moroney et al., 1986). It is likely that both the CCM and the C2 cycle must be functional for algal cells to successfully adapt to low-CO_2 conditions.

A model of the CCM in *Chlamydomonas reinhardtii* has been proposed (Moroney and Mason, 1991), and in this model the periplasmic space, the plasma membrane, the chloroplast envelope, and the pyrenoid are key sites of carbon uptake or assimilation. In *C. reinhardtii* the CCM is inducible. In vivo labeling of *C. reinhardtii* with $^{35}SO_4^{2-}$ demonstrated the presence of five proteins that were synthesized preferentially on low CO_2 (Coleman and Grossman, 1984; Manuel and Moroney, 1988; Spalding and Jeffrey, 1989). One of these proteins is a 37-kD periplasmic carbonic anhydrase (Coleman and Grossman, 1984), and its gene has been cloned and sequenced (Fukuzawa et al., 1990). Another low-CO_2-induced protein is a phosphoglycolate phosphatase (Marek and Spalding, 1991), which is the second enzyme of the C2 cycle (Husic et al., 1987). The onset of biosynthesis of these proteins closely matches the onset of the CCM. Some *C. reinhardtii* mutants that cannot grow at low-CO_2 conditions fail to induce one or more of these low-CO_2-inducible peptides (Moroney et al., 1989; Katzman et al., 1994).

Under the assumption that some of these peptides may be required for growth on low CO_2, a cDNA library was made from cells that had adapted to low CO_2 for 2 h. Through differential screening, six classes of noncross-hybridizing, low-CO_2-inducible clones were isolated (Burow et al., 1996). Here we report the biochemical and molecular characterization of an Ala AT gene that is induced by low-CO_2 conditions. We have also investigated the induction of Ala AT enzyme activity during the adaptation to low-CO_2 conditions and its possible involvement in the CCM or C2 cycle in *C. reinhardtii*.

MATERIALS AND METHODS

Cell Culture

Wild-type *Chlamydomonas reinhardtii* 137^+ was originally obtained from Dr. R.K. Togasaki (Indiana University, Bloomington). For growth in liquid culture cells were first inoculated from yeast-acetate medium (Sueoka, 1960) plates to 100 mL of Tris-acetate-phosphate liquid medium with continuous shaking and light (300 μE m^{-2} s^{-1}) for 2 d.

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Abbreviations: Ala AT, Ala aminotransferase; AOA, aminooxyacetic acid; CCM, CO_2-concentrating mechanism; Chl, chlorophyll; $K_{0.5}(CO_2)$, the CO_2 concentration required to give one-half of the maximal photosynthetic rate; RACE, rapid amplification of cDNA ends.

The culture was then transferred to a flask with 1.5 L of minimal medium (Sueoka, 1960) and aerated with 5% CO_2 until it reached a density of 1×10^5 cells/mL. The culture was diluted with an equal volume of fresh medium and split into two flasks: one aerated with the air containing 5% CO_2, and the other bubbled with the air containing 0.035% CO_2 (low CO_2). The time of air adaptation depended on the experiments, varying from 1 to 10 h. After cells were switched to low CO_2 in the presence of AOA, an inhibitor of Ala AT (Tolbert et al., 1983), they were harvested and washed twice with fresh medium to remove AOA before being resuspended in 25 mM Hepes-KOH (pH 7.3) to measure O_2 evolution.

RNA Purification and Analysis

All of the utensils were either baked at 180°C overnight or soaked with diethyl pyrocarbonate-treated water and autoclaved for 2 h. The total RNA was extracted from *C. reinhardtii* using standard procedures (Sambrook et al., 1989). For RACE experiments, an mRNA isolation system (Poly AT tract, Promega) was used to purify mRNA from total RNA that was isolated from cells adapted to air for 2 h. For northern blot analysis, denatured RNA was resolved on 1.0% agarose/6% formaldehyde gels and transferred to nitrocellulose membranes (BA-S 85, Schleicher & Schuell) after the gel was soaked overnight in diethyl pyrocarbonate-treated water (Sambrook et al., 1989). The northern blot was probed with 4I29 S, a short clone of 4I29 that has a 0.6-kb insert corresponding to the 3′ end of 4I29 clone. The final washing conditions were 0.5× SSC (75 mM NaCl, 7.5 mM sodium citrate) + 0.1% SDS at 55°C for 2 h or longer.

DNA Preparation and Sequencing

Plasmid DNAs were prepared by alkaline extraction (Sambrook et al., 1989). Before sequencing, double-stranded plasmid DNAs were purified by Glassmilk (Bio 101, Vista, CA) using the procedures provided by the manufacturer. Single-stranded DNA was isolated as described (Sambrook et al., 1989) using the m13k07 helper phage (Bio-Rad). Either 1 μg of single-stranded DNA or 3 μg of purified double-stranded DNA was sequenced using the United States Biochemical Sequenase kit according to the manufacturer's instructions. Sequence comparisons were made using the Blast (Altshul et al., 1990) or Blitz programs (Biocomputing Research Unit, Edinburgh, UK) via e-mail servers.

Cloning of 5′ End cDNA by RACE

The RACE procedure used to clone the 5′ end of 4I29 was a modification of earlier methods (Frohman, 1990; Dorit et al., 1993). One microliter of 10 μM RACE primer C (5′-AGAAGTTTGAGGC AGTAGAA- 3′) and 1 μL of 200 mM Tris-HCl, pH 7.5, were added to 1 μg of mRNA (in 4 μL of water) and denatured by heating at 70°C for 10 min. This reaction mixture was snap-chilled on ice for 3 min, and the contents were collected by a quick spin. The mRNA and the primer in the reaction mixture were then annealed at 45°C for 2 h. To this annealed mixture, 4 μL of 5× first-strand buffer (250 mM Tris-HCl, pH 8.3, at room temperature, 375 mM KCl, 15 mM $MgCl_2$), 2 μL of 0.1 M DTT, 1 μL of mixed deoxyribonucleotide triphosphated stock (10 mM for each, pH 7.0), and 6 μL of water were added and mixed by a gentle spin; the tube was prewarmed at 45°C for 2 min. One microliter of Moloney murine leukemia virus Superscript II (200 units/μL, BRL) was added and incubated at 45°C for 1 h. The reaction was terminated by adding 4 μL of 0.5 M EDTA, diluted to 2 mL with 0.1 × TE (1 × TE = 10 mM Tris-HCl, 1 mM EDTA, pH 7.5), and spun at 1000*g* for 20 min in a cartridge (Centricon-100, Amicon, Beverly, MA) to remove excessive primers and nucleotides; the above dilution and spin were repeated once, and the retentate was collected and concentrated to 10 μL on a speed-vacuum (Savant Instruments, Farmingdale, NY).

To 5 μL of the above retentate (first-strand cDNA), 2 μL of 5× terminal deoxynucleotide transferase buffer (500 mM potassium cacodylate, pH 7.2, 10 mM $CoCl_2$), 1 μL of 2 mM dATP, 1 μL of water, and 1 μL of terminal deoxynucleotide transferase (18 units/μL) were added. The tailing reaction mixture was incubated at 37°C for 30 min and inactivated by heating at 65°C for 10 min.

Two rounds of PCR amplifications were usually performed. For the first-round PCR, the reaction volume was 100 μL, including 1× reaction buffer (50 mM Tris-HCl, pH 9.0, 20 mM ammonium sulfate), 2 mM $MgCl_2$, 150 nM (each) deoxyribonucleotide triphosphate, 100 nM RACE primer C, 40 nM RSdT primer (5′-GAGAGAGAGAGAGAGAGA-ACTAGTCTCGAGTTTTTTTTTTTTTTTT-3′), 1.5% formamide, 1 μL of 1:20 diluted and tailed first-strand cDNA, and 0.3 unit of *Tfl* DNA polymerase (Epicenter, Madison, WI). PCR profiles were: 1 cycle at 97°C for 5 min, 42°C for 3 min, and 72°C for 25 min; 35 cycles at 95°C for 40 s, 53°C for 1 min, 72°C for 2.5 min; 1 cycle at 72°C for 15 min. For second-round PCR, the reaction mixture was the same except that the RACE primer C was replaced with RACE primer B (5′-TTAATCTGGGGGAAGCTGTACAT-3′) and the DNA amplified from the first-round PCR was substituted for the first-strand cDNA. The PCR conditions were the same as in the first round, except that the first cycle was skipped.

The PCR mixture was resolved on a 0.8% agarose gel, and the amplified DNA band was recovered and subsequently cloned into a cloning vector (pCRII, Invitrogen, San Diego, CA) according to the manufacturer's procedure. One of the clones harboring the longest 5′ end of 4I29 (1.6 kb) was named 4I29 L.

Ala AT and Asp Aminotransferase Activity Assays

Cells were homogenized at 2000 p.s.i. in a Parr bomb (Parr Instrument, Moline, IL) in extraction buffer (0.1 M Tris-HCl, pH 8.0, containing 10 mM DTT, and 10% [v/v] glycerol). Ala AT activity was assayed in the direction from Ala to pyruvate, which was further converted to lactate concomitant with the oxidation of NADH by lactate dehydrogenase (Muench and Good, 1994). The Asp AT was assayed in the direction from Asp to oxaloacetate, which was then converted to malate by malic dehydrogenase

(M9004, Sigma) with the oxidation of NADH. The oxidation of NADH was monitored spectrophotometrically at 340 nm at room temperature (23°C). All of the solutions were made in 0.1 M Tris-HCl buffer except lactate dehydrogenase (L-1254, Sigma), which was dissolved in 1 mL of 10 mM potassium phosphate (pH 7.5) and 50% glycerol and stored at 4°C.

Ser:Glyoxylate Aminotransferase Activity Assay

Ser:glyoxylate aminotransferase was assayed using ^{14}C-labeled Ser, and the formation of [^{14}C]hydoxypyruvate was counted after separation of Ser and hydroxypyruvate by TLC. The procedure was modified from Izumi et al. (1990) and Benson et al. (1950). The reaction was performed in 600 μL at 31°C with the following reagents: 60 μL of 200 mM Ser (3-^{14}C labeled, specific activity 125 μCi mmol^{-1}), 30 μL of 100 mM sodium glyoxylate, 3.0 μL of 20 mM pyridoxal phosphate, 150 μL of cell extract (1 mg Chl mL^{-1}), and 257 μL of 0.1 M potassium phosphate buffer (pH 7.0). The reaction was started by adding Ser, and a 100-μL aliquot was taken out at 0, 1, 2, 5, and 10 min. The reaction was stopped by heating at 95°C for 3 min. The tubes containing the aliquots were spun at 15,000*g* for 10 min to pellet the cell debris, and 40 μL of supernatant from each tube was transferred to another set of tubes. The supernatant was concentrated to 10 μL, and 2 μL of the concentrated sample from each time point was applied to the TLC (20 cm × 20 cm). The solvent used to separate the radioactive Ser and hydroxypyruvate was phenol:acetic acid:water (8:1:1; v/v); the separation usually took 6 to 8 h. After air-drying, the control Ser lane was visualized by spraying with 0.1% ninhydrin, and the control hydroxypyruvate lane was visualized by spraying with 6 N sulfuric acid, followed by heating the TLC plate at 120°C for 2 to 5 min. The spot in the untreated lanes corresponding to hydroxypyruvate was then recovered, and the radioactivity was counted by a scintillation counter (model LS-8000, Beckman).

Other Methods

For sequencing purposes, nested deletions of clone 4I29 L were made according to Sambrook et al. (1989). Photo-

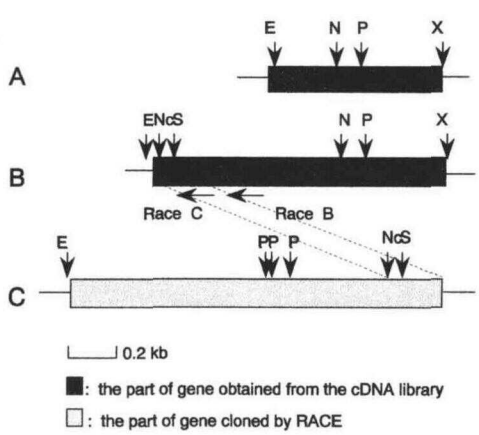

Figure 1. The restriction maps of different 4I29 clones. A, 4I29 S; B, 4I29 M; C, 4I29 L. The restriction sites E, N, Nc, P, S, and X indicate *Eco*RI, *Nsi*I, *Nco*I, *Pst*I, *Stu*I, and *Xho*I, respectively.

Figure 2. Northern blot analysis of the expression of clone 4I29. The high-CO_2-grown cells were switched to low-CO_2 conditions at 0 h. Total RNA was isolated from 0-, 4-, and 10-h CO_2-grown cells (indicated by a C in the lanes), and from 1-, 2-, 4-, 6-, and 10-h low-CO_2-adapting cells (indicated by an A in the lanes).

synthetic CO_2-dependent O_2 evolution was measured in an O_2 electrode (Rank Brothers, Cambridge, UK) (Badger et al., 1980; Moroney and Tolbert, 1985). The accumulation of inorganic carbon by the algal cells was estimated by centrifugation through silicone oil as previously described (Badger et al., 1980; Moroney and Tolbert, 1985; Moroney et al., 1989). Chl concentrations were determined spectrophotometrically after extraction with 100% methanol. Glycolate excretion was determined using the method of Calkins (1943). All of the assays were repeated at least three times.

RESULTS

Clone 4I29 Induction after Switching to Low-CO_2 Conditions

After differential screening of the cDNA library, six classes of low-CO_2-inducible clones were selected (Burow et al., 1996). One clone, 4I29 S, with a cDNA insert of 0.6 kb was chosen for further study (Fig. 1). First, the expression of the 4I29 clone in air- and CO_2-grown cells was compared. The total RNA was isolated from CO_2-grown cells and cells that had been adapted to air for 1, 2, 4, 6, and 10 h. A northern blot of RNA samples over the time course was probed as described in "Materials and Methods." The clone 4I29 recognized an mRNA of about 2.6 kb, and the results confirmed that it was strongly induced after the cells were switched to low-CO_2 conditions. The mRNA level of clone 4I29 increased 4- to 5-fold within 2 h after the culture was switched from high to low CO_2. After 4 to 6 h, the amount of 4I29 mRNA decreased slowly (Fig. 2). The mRNA corresponding to 4I29 also was detected in RNA samples from high-CO_2-grown cells, indicating that this gene is expressed constitutively at low levels in high-CO_2-grown cells (Fig. 2).

Cloning the 5′ End of Ala AT cDNA by RACE

To obtain the full-length Ala AT cDNA, two synthetic RACE primers (B and C) were made. mRNA that was isolated from cells that had adapted to air for 2 h was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase using RACE primer C, and the first-

strand cDNA was tailed with poly(A). After two rounds of PCR amplification, a 1.6-kb band was visible on an agarose gel. This DNA fragment was recovered and subsequently cloned into the pCRII cloning vector (Invitrogen). The vector containing this 1.6-kb insert was named 4I29 L (Fig. 1).

Complete DNA Sequence and Amino Acid Sequence Homology Comparison

Both strands of clones 4I29 L, 4I29 S, and 4I29 M were sequenced. The complete cDNA of Ala AT is 2552 bp long, and it has a coding region of 521 amino acids with a deduced molecular weight of 58,000. The DNA sequence showed significant homology with Ala AT-2 from *Panicum miliaceum* (Son and Sugiyama, 1992), barley (Muench and Good, 1994), humans (Ishiguro, et al., 1991a), and rats (Ishiguro et al., 1991b). It also showed some homology to ACC synthase, which functions in catalyzing the formation of ethylene from ACC in plants. No homology was found with any known Ala:glyoxylate aminotransferase. The deduced protein sequence of Ala AT from *C. reinhardtii* has 49.4% identity with that from *P. miliaceum*, and 47.9, 46.3, and 45.0% identity with Ala AT from barley, humans, and rats, respectively (Fig. 3). The western blot using the antibody raised against barley Ala AT-2 (a kind gift from Dr. Allen G. Good, University of Alberta, Edmonton) identified a 53-kD single protein band from both air- and CO_2-grown cells, and the level of this 53-kD protein increased when cells were switched from high-CO_2 to low-CO_2 conditions, to about 4-fold after 6 h (Fig. 4). This agrees very well with the results of northern blot analysis and DNA

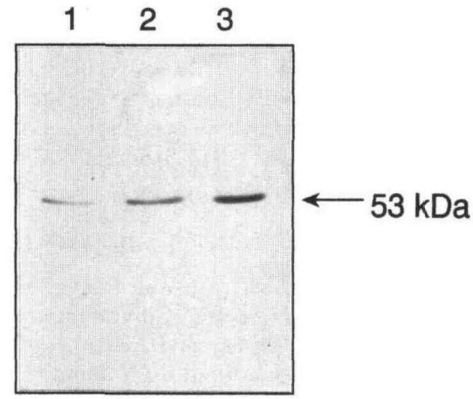

Figure 4. Western blot analysis of Ala AT at high-CO_2 and low-CO_2 growth conditions. Lane 1, *C. reinhardtii* cells grown at high-CO_2 conditions; lane 2, cells that have been adapting to air for 2 h; lane 3, cells that have been adapting to air for 6 h. Twenty micrograms of total protein was loaded per lane.

sequence homology comparison, and it further confirmed that the clone 4I29 encodes an Ala AT.

Induction of Ala AT Activity in *C. reinhardtii* Cells after Transfer from High-CO_2 to Low-CO_2 Conditions

The Ala AT activity was monitored to determine whether the induction of Ala AT at the mRNA level correlates with an increase in enzyme activity. The Ala AT activity was assayed in both high-CO_2-grown cells and low-CO_2-adapting cells every 2 h up to 12 h after the

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Barley   --------MAATVAV DN---------------- ---------------- -------------LN PKVLKCEYAVRGEIV IHAQRLQEQLKTQPG     41
Panicum  --------MAATVAV EN---------------- ---------------- -------------LN PKVLKCEYAVRGEIV IHAQHLQQQLQTQPG     41
C.r.     MRKEATRLVSALLRA GNNGVSTSWAVGGTR LKSAMPQPDEKKDED LHAKEGKVLHPHLLN ENVVKTQYAVRGELY LRAEQLRKEGKE---     87
Rat      ---MASRVNDQSQAS RNG------------ ---------------- ---LKGKVLTLDTMN PCVRRVEYAVRGPIV QRALELEQELRQG-V     56
Human    ----ASSTGDRSQAV RHG------------ ---------------- ---LRAKVLTLDGMN PRVRRVEYAVRGPIV QRALELEQELRQG-V     55
                                                                  *   *    *****      *  *

Barley   SLPFDEILYCNIGNP QSLGQQPVTFFREVL ALCDHPDLLQREEIK TLFSADSISRAKQIL AMIPGRATGAYSHSQ GIKGLRDAIASGIAS     131
Panicum  SLPFDEILYCNIGNP QSLGQQPVTFFREVL ALCDHPCLLEKEETK SLFSADAISRAKQIL STIPGRATGAYSHSQ GIKGLRDAIAAGIAS     131
C.r.     ------IIFTNVGNP HALGAKPLTFTRQVL ALCAAPFLLDHPKVE DMFPADAIARAKKIL ASFKG-GVGAYTDSR GNPLVREEVARFIEK     170
Rat      KKPFTEVIRANIGDA QAMGQRPITFFRQVL ALCVYPNLLSSP--D --FPEDAKRRAERIL QACGGHSLGAYSISS GIQPIREDVAQYIER     142
Human    KKPFTEVIRANIGDA QAMGQRPITFLRQVL ALCVNPDLLSSP--N --FPDDAKKRAERIL QACGGHSLGAYSVSS GIQLIREDVARYIER     141
                  * *      *  * ** * ** *** * **          *  *   **  **     *   *** *  *    *       *

Barley   RDG-FPANADDIFLT DGASPGVHLMMQLLI RNE---KDGILVPIP QYPLYSASIALHGGA LVPYYLNESTGWGLE TSDVKKQLEDARSRG     217
Panicum  RDG-FPANADDIFVT DGASPGVHMMMQLLI RNE---KDGILCPIP QYPLYSASIALHGGT LVPYYLDEKTGWGLE ISDLKKQLEDARSKG     217
C.r.     RDG-VPSNPDHIFLT DGASVAVRLCLNAMI RHD---RDSVLVPIP QYPLYSASIRLYGGT LVGYFLDERRGWGLS VEELQRALQESREEG     256
Rat      RDGGIPADPNNIFLS TGASDAIVTMLKLLV SGEGRARTGVLIPIP QYPLYSAALAELDAV QVDYYLDEERAWALD IAELRRALCQARDR-     231
Human    RDGGIPADPNNVFLS TGASDAIVTVLKLLV AGEGHTRTGVLIPIP QYPLYSATLAELGAV QVDYYLDEERAWALD VAELARALGQARDH-     230
         ***  *      *   ***                      * *** *******         * * * *   * *          *   *

Barley   INVRALVVINPGNPT GQVLAEENQYDIVKF CKNEGLVLLADEVYQ ENIYVDNKKFHSFKK IVRSLGYGE-EDLPL VSYQSVSKGYYGECG     306
Panicum  IDVRALVVINPGNPT GQVLAEDNQCDIVRF CKNEGLVLLADEVYQ ENIYVDDKKFNSFKK IARSVGYGE-DDLPL VSFQSVSKGYYGECG     306
C.r.     KLVRGLVFINPGNPT GQCLSKENLQELIKL AYQEKIVLMADEVYQ ENVYQDERPFVSAKK VMWEMGEPYRSHVEL LSFHTVSKGTAGECG     346
Rat      CCPRVLCVINPGNPT GQVQTRECIEAVIRF AFKEGLFLMADEVYQ DNVYAEGSQFHSFKK VLMEMGPPYSTQQEL ASFHSVSKGYMGECG     321
Human    CRPRALCVINPGNPT GQVQTRECIEAVIRF AFEERLFLLADEVYQ DNVYAAGSQFHSFKK VLMEMGPPYAGQQEL ASFHSTSKGYMGECG     320
           *  *  ******* **                 *   * ******  * *    * * **      *         *  *    ***  ****

Barley   KRGGYFEITGFSAPV REQIYKIASVNLCSN ITGQILASLVMNPPK ASDESYASYKAEKDG ILASLARRAKALEHA FNKLEGITCNEAEGA     396
Panicum  KRGGYMEITGFSAPV REQIYKIASVNLCSN ITGQILASLVMNPPK VGDESYAAYKAEKDG ILQSLARRAKALEDA FNNLEGISCNKAEGA     396
C.r.     LRGGYVEMTNIHPGA IEEVYKCASINLSPN TMGQIALSVLVNPPK PGDPSYDQYTKEKAS ELVSLRRRRHMVTDG FNALDGVTCNFTEGA     436
Rat      FRGGYVEVVNMDAEV QKQMGKLMSVRLCPP VPGQALMDMVVSPPT PSEPSFKQFQAERQE VLAELAAKAKLTEQV FNEAPGIRCNPVQGA     411
Human    FRGGYVEVVNMDAAV QQQMLKLMSVRLCPP VPGQALLDLVVSPPA PTDPSFAQFQAEKQA VLAELAAKAKLTEQV FNEAPGISCNPVQGA     410
          **** *              *  *  *     **          **     *       *   *                **   *  **   **

Barley   MYVFPQICLPQKAIE AAKAANKAPDAFYAL RLLESTGIVVVPGSG FGQVPGTWHFRCTIL PQEDKIPAVISRFTV FHEAFMSEYRD         482
Panicum  MYLFPQIHLPKKAIE AAKAANKAPDAFYAL RLLESTGIVVVPGSG FGQVPGTWHIRCTIL PQEDKIPAVITRFKA FHEAFMAEYRD         482
C.r.     MYSFPQIKLPAKALE AAKAAGKAGDVFYCL KLLEATGISTVPGSG FGQEEGTFHLRTTIL PREEVMTTFVEKFDK FHKDFMKQYS-         521
Rat      MYSFPQVQLPLKAVQ RAQELGLAPDMFFCL CLLEETGICVVPGSG FGQQEGTYHFRMTIL PPMEKLRLLLEKLSH FHAKFTHEYS-         496
Human    MYSFPRVQLPPRAVE RAQELGLAPDMFFCL RLLEETGICVVPGSG FGQREGTYHFRMTIL PPLEKLRLLLEKLSR FHAKFTLEYS-         495
         ** **   **  *    *     * * *  *  *** ***  *****  ***  ** * * *** *              **  *   *
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Figure 3. Protein sequence alignment of Ala AT from barley, *P. miliaceum*, *C. reinhardtii*, rat, and human. Asterisks (*) indicate residues that are common to all five sequences.

culture was switched from high to low CO_2 (Fig. 5). The Ala AT activity increased 4-fold in 6 h from 60 to 240 μmol NADH mg^{-1} Chl h^{-1} after transfer to low CO_2. In contrast, Ala AT activity from cells grown at high-CO_2 conditions fluctuated only slightly (Fig. 5).

The Presence of AOA Significantly Decreased the Maximum Rate of Photosynthesis in Air-Adapting Cells

To determine the physiological role of Ala AT at low-CO_2 conditions, we added the aminotransferase inhibitor AOA to cells during and after the adaptation to low CO_2. The sensitivities of Ala AT and other aminotransferases such as Asp aminotransferase and Ser:glyoxylate aminotransferase were studied first using in vitro assays. The addition of 10 μM AOA in the activity assay buffer inhibited 95% of Ala AT activity, whereas 6 μM inhibited about 90%. Under the same conditions, 10 μM AOA had little effect on the activities of Asp AT and Ser:glyoxylate aminotransferase. The addition of 40 μM AOA to the assay buffer inhibited 97.2% of Ala AT, 70.4% of Asp aminotransferase, and 22% of Ser:glyoxylate aminotransferase activities (Table I). When 40 μM AOA was added to cells for 30 min before harvesting, the activity of Ala AT in the cell homogenate was inhibited more than 95%, whereas the Asp aminotransferase was inhibited only 55%, and Ser:glyoxylate aminotransferase activity was inhibited about 16%. These results suggest that 40 μM AOA can be used to study the possible functions of Ala AT without causing serious side effects on other metabolic pathways.

To determine the possible physiological roles of Ala AT, AOA was added to a final concentration of 40 μM in the growth medium when cells were beginning to adapt to low-CO_2 growth conditions. For control cells left at high-CO_2 growth conditions, AOA was added at the same time. The CO_2-dependent photosynthetic O_2 evolution activity

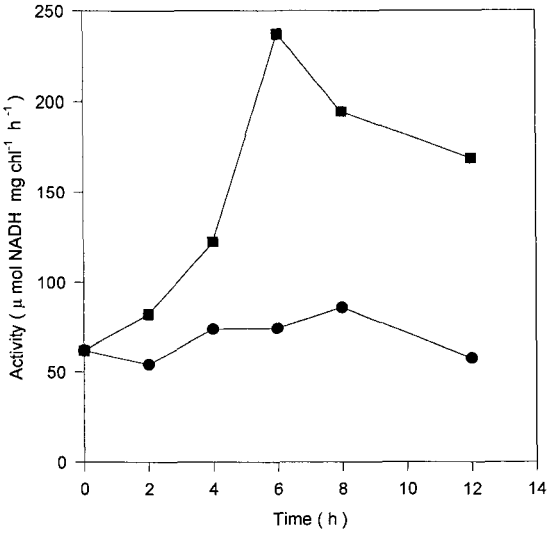

Figure 5. The induction of Ala AT activity by low-CO_2 growth conditions. Ala AT activity was measured after switching cells to low-CO_2-growth conditions (■) for the indicated amount of time, compared with cells left at high-CO_2 conditions (●).

Table I. *The sensitivities of aminotransferases to AOA in the cell homogenate of C. reinhardtii grown at low CO_2*

The enzyme activities were assayed in the presence or absence of 40 μM AOA in the reaction buffer.

Protein	Activity		Inhibition
	−AOA	+AOA	
	μmol mg^{-1} Chl h^{-1}		*%*
Ala AT	288.0	8.1	97.2
Asp aminotransferase	138.3	41.0	70.4
Ser/Glyoxylate aminotransferase	31.0	24.2	22.0

was assayed 6 to 8 h after transfer. At this time, Ala AT reached its highest activity (Fig. 5). The addition of AOA to the growth medium had little effect on the maximum photosynthetic rate when cells were still growing at high-CO_2 conditions (Fig. 6); however, AOA lowered the maximum rate of photosynthesis from 260 to 175 μmol O_2 mg^{-1} Chl h^{-1} when it was added to cells that were adapting or had adapted to low-CO_2 growth conditions (Fig. 6). Therefore, in the presence of AOA, which inhibited Ala AT activity, low-CO_2-grown cells were not able to reach their maximum rate of photosynthesis even though normally saturating levels of external HCO_3^- were provided.

AOA Decreased Inorganic Carbon Uptake in Air-Adapting Cells

A more direct way of studying the relation between Ala AT and the CCM is to measure the inorganic carbon uptake while inhibiting the Ala AT by AOA. High-CO_2-grown cells and cells that had been adapted to air with or without 40 μM AOA for 8 h were harvested for the inorganic carbon uptake studies. Cells were washed twice with fresh me-

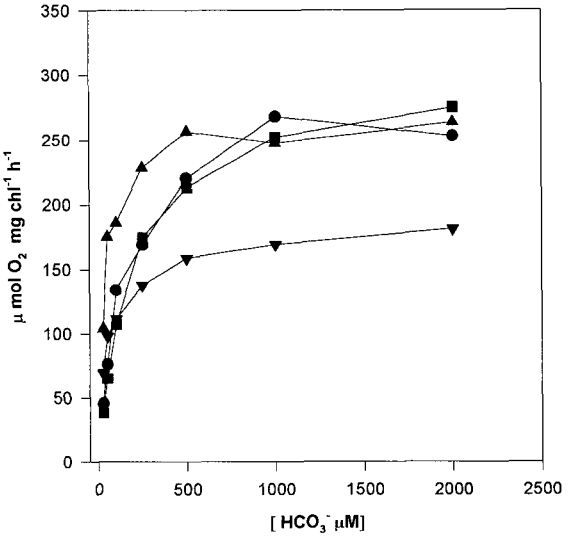

Figure 6. Effect of AOA on photosynthetic O_2 evolution. High-CO_2-grown cells (●, ■) or air-adapting cells (▲, ▼) that have been treated with AOA (40 μM) (■, ▼) or without AOA (●, ▲) for 8 h. Cells were washed after harvest with O_2 evolution buffer (25 mM Hepes-KOH, pH 7.3), and photosynthetic O_2 evolution was determined at varying inorganic carbon concentrations.

dium before being resuspended in N_2-bubbled 25 mM Hepes-KOH (pH 7.3); this is the buffer used to determine O_2 evolution in vivo. For cells that had adapted to air in the presence of 40 μM AOA, the internal inorganic carbon concentration was 0.45 mM at 60 s and 0.69 mM at 90 s, whereas in the untreated cells it was 1.19 and 1.34 mM, respectively (Fig. 7). The overall inorganic carbon uptake in AOA-treated air-grown cells was about 50% slower. For high-CO_2-grown cells, the difference in inorganic carbon uptake was not significant (Fig. 7), although inorganic carbon uptake by high-CO_2-grown cells is low with or without AOA.

Short-Term Effects of AOA

Although the inorganic carbon uptake studies are consistent with the hypothesis that the Ala AT may play a role in the CCM, the short-term effects of AOA on inorganic carbon affinity were minimal. When 40 μM AOA was added to fully adapted low-CO_2 cells for short periods of time (a few seconds to 30 min) we were unable to measure any significant change in the affinity of the cells for external inorganic carbon even though Ala AT was almost completely inhibited by this treatment (Table II). Even cells treated with 1 mM AOA for less than 5 min had nearly maximal levels of photosynthesis and normal affinities for inorganic carbon (data not shown), although a 30-min exposure to 1 mM AOA did inhibit photosynthesis (Table II). In contrast, cells treated with low levels of AOA excreted glycolate at high rates in a short period of time (Table III). The rates of glycolate excretion by cells treated with 40 μM AOA are similar to rates reported previously (Tolbert et al., 1983; Moroney et al., 1986) and are close to the estimated rate of flux of the C2 cycle when cells are incubated in the light with air levels of CO_2 and O_2 (Moroney et al., 1986).

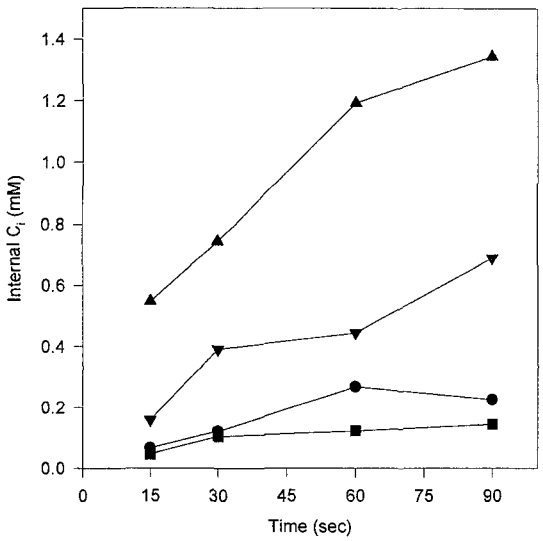

Figure 7. Inorganic carbon uptake in cells treated with AOA. The effect of AOA (40 μM) on inorganic carbon uptake of CO_2-grown cells (●, ■) versus air-adapting cells (▲, ▼) that have been treated with AOA (■, ▼) or without AOA (●, ▲) for 8 h. Cells were washed after harvest with O_2 evolution buffer (25 mM Hepes-KOH, pH 7.3).

Table II. *The effect of AOA on the affinity of low-CO_2-grown cells for external inorganic carbon*

Cells were grown in minimal medium under low-CO_2 conditions, and AOA was added to the illuminated culture where indicated. The affinity of the cells for inorganic carbon was estimated by determining the concentration of CO_2 required for one-half-maximal rates of CO_2-dependent O_2 evolution or the $K_{0.5}(CO_2)$. Ala AT activity was measured as described in "Materials and Methods."

Treatment	Ala AT Activity	$K_{0.5}(CO_2)$	V_{max}
	μmol NADH mg^{-1} Chl h^{-1}	*μM*	*μmol O_2 mg^{-1} Chl h^{-1}*
Untreated	144.0	2.5	164
40 μM AOA, 30 min	10.7	3.5	162
1 mM AOA, 30 min	3.6	6.8	135

From Tables II and III, it appears that cells treated with 40 μM AOA excrete glycolate at high rates (Table III) but retain their apparent high affinity for external inorganic carbon (Table II).

DISCUSSION

Induction of Ala AT

C. reinhardtii induces a number of genes and synthesizes specific proteins when placed in a low-CO_2-containing environment. In this paper we show that one of these inducible genes is an Ala AT. We have demonstrated that low-CO_2-grown *C. reinhardtii* has a very high level of Ala AT activity (>200 μmol h^{-1} mg^{-1} Chl). We have also confirmed that Ala AT activity increases significantly when cells are switched from high to low CO_2. The activity increase (Fig. 5) parallels the increase in the mRNA encoding the Ala AT when cells are switched to low CO_2 (Fig. 2).

The Effect of AOA

Tolbert et al. (1983) showed that short-term exposure of *C. reinhardtii* cells to 1 mM AOA caused them to excrete glycolate but did not inhibit photosynthetic CO_2 fixation. In their experiments 1 mM AOA treatment of air-grown *C. reinhardtii* also resulted in the complete blockage of [^{14}C]Ala formation and a nearly corresponding increase of [^{14}C]pyruvate when cells were illuminated in the presence of low levels of $^{14}CO_2$. AOA blocked Ala formation by *C. reinhardtii* grown on high CO_2, although pyruvate accumu-

Table III. *Glycolate excretion by cells treated with AOA*

Low- or high-CO_2-grown cells were harvested and resuspended in 150-mL flasks at 25 mg Chl/mL with fresh media. The flasks were bubbled with air under light and continuous stirring for 30 min in the presence (+) or absence (−) of 40 μM AOA. The cells were then removed by centrifugation, and the supernatants were used for the glycolate assay (Calkins, 1943).

Growth Conditions	AOA Treatment	Glycolate Excretion
		μmol mg^{-1} Chl h^{-1}
Low CO_2	−	0.4
Low CO_2	+	16.4
High CO_2	−	7.4
High CO_2	+	12.4

lation was not noted, but it did not block the biosynthesis of Asp or Glu during photosynthesis by *C. reinhardtii*, which would also have involved aminotransferase reactions (Tolbert et al., 1983). However, AOA did not inhibit the enzymes associated with glycolate synthesis from ribulose-1,5-bisphosphate. Neither did 1 mM AOA inhibit the carboxylase or oxygenase activities of purified Rubisco from spinach leaves, or P-glycolate phosphatase, glycolate dehydrogenase, or carbonic anhydrase from the alga (Tolbert et al., 1983). It is evident from our studies that Ala AT is very sensitive to AOA inhibition (Fig. 6). Further, the concentration of AOA used in this study was only 40 μM, much lower than 1 mM, to keep the possible side effects caused by AOA to a minimum.

In the present experiments, since the O_2 evolution activity was expressed on the basis of milligrams of Chl, the decrease of V_{max} in cells adapting to low-CO_2 conditions in the presence of 40 μM AOA might be simply because AOA resulted in more dead cells, very slow growth, or little new protein biosynthesis at low-CO_2 conditions. The effect of AOA on these aspects was investigated. We found no increase in cell death due to AOA. In addition, the decrease in cell growth rate caused by AOA in low-CO_2 cells and high-CO_2-grown cells for the first 8 h was similar (Z.-Y. Chen and J. V. Moroney, unpublished results). An additional possibility was that AOA blocked different aminotransferases to different extents and limited the availability of free amino acids for the biosynthesis of new proteins, which is required for the establishment of the CCM. To check this possibility, cells were switched to low CO_2 in the presence or absence of AOA and probed for the presence of the low-CO_2-inducible protein LIP-36 (Ramazanov et al., 1993). We found that LIP-36 was made in nearly normal amounts, indicating that 40 μM AOA was not grossly interfering with protein biosynthesis.

Possible Physiological Role(s) of Ala AT

The data presented in this report are consistent with the assumption that Ala AT is required for the successful adaptation of *C. reinhardtii* to low-CO_2 growth conditions. The level of the mRNA encoding Ala AT is increased under low-CO_2 conditions, and AOA inhibits photosynthesis when cells are grown under low-CO_2 conditions but has no effect when cells are grown under elevated CO_2 (Fig. 6). One possible physiological role for Ala AT is that it is required for the efficient operation of the C2 cycle. In this role it would be part of the shuttle of amino groups from Glu to glyoxylate. A second potential role is that Ala AT has a role in the CCM of *C. reinhardtii* possibly by shuttling C3 equivalents, which is seen in some higher plants with NAD-malic-type C4 metabolism (Hatch, 1987). The data presented in this report are consistent with the hypothesis that Ala AT plays a role in the C2 cycle in *C. reinhardtii*.

One argument in favor of a C2-cycle role for the Ala AT is the observation that the increase in mRNA levels is transient, occurring during the first few hours after transfer of the cells to low CO_2. After 6 h on low CO_2, the amount of Ala AT mRNA is very similar to that of cells growing on elevated CO_2 (Fig. 2). If the Ala AT were an integral part of the CCM, one might expect that the level of its mRNA would remain elevated as long as the cell was under low-CO_2 conditions; however, this was not the case. We also found that 40 μM AOA caused glycolate excretion by low-CO_2-adapted cells (Table III), which could not be explained by the inhibition effect of AOA on Ser:glyoxylate aminotransferase (Table I). This result is consistent with Ala AT being essential to the efficient operation of the C2 cycle. It is possible that an inhibition of the CCM would also result in an increase in the formation of glycolate. However, unless 40 μM AOA also inhibits the C2 cycle, one would not expect this to cause an excretion of glycolate. An additional argument against Ala AT playing a direct part in the CCM is that in short exposures (<30 min), AOA had no effect on the maximal rate of photosynthesis or on the inorganic carbon affinity exhibited by the cells (Table II) even when the Ala AT was completely inhibited. Only when the cells were treated with AOA for long periods of time did an effect on the maximal rate of photosynthesis become apparent. This inhibition after a long-term treatment with AOA is consistent with Ala AT acting as part of the shuttle of amino groups from Glu to glyoxylate. At atmospheric levels of CO_2 and O_2, the flux of carbon through the C2 cycle is low compared with the C3 cycle (10 versus 140 μmol h^{-1} mg^{-1} Chl, respectively) in low-CO_2-grown *C. reinhardtii* (Moroney et al., 1986). At these rates, the effect of inhibiting the C2 cycle would be minor at first, but would cause an imbalance over a period of time when the cells are unable to efficiently cycle the carbon entering the C2 cycle. The inhibition of photosynthesis observed after a 6- to 8-h treatment with AOA is consistent with this long-term effect expected if the C2 cycle were blocked. Since the metabolite flux through the C2 cycle is minimal when cells have high CO_2 concentrations in the environment, AOA does not inhibit photosynthesis in cells growing on elevated CO_2. An additional argument against Ala AT playing a direct role in the CCM is that even after a long-term inhibition we found no evidence that 40 μM AOA altered the affinity of the cells for inorganic carbon, as estimated by the $K_{0.5}(CO_2)$. If Ala AT was essential to the operation of the CCM there should have been a large increase in the $K_{0.5}(CO_2)$ when AOA was added to the cells.

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